FUNTIONAL CHARACTERIZATION OF VACUOLAR ATP-BINDING CASSETTE TRANSPORTERS IN *CANDIDA ALBICANS*

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FUNCTIONAL CHARACTERIZATION OF

VACUOLAR ATP-BINDING CASSETTE

TRANSPORTERS IN *CANDIDA ALBICANS*

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Abstract:
ATP binding cassette transporters are a large family of integral membrane transport proteins which have vital functions in prokaryotic and eukaryotic organisms. The genome of *Candida albicans*, the most important opportunistic fungal pathogen in humans, harbors more than ten ABC transporter genes belonging to four ABC transporter subfamilies. In previous studies, we have characterized the Mlt1 ABC transporter, a member of the multidrug resistance-associated protein (MRP) subfamily of ABC transporters. Ycf1 (yeast cadmium factor 1), another member of this subfamily, initially was found in *Saccharomyces cerevisiae* sharing 42.6% identity with human MRP1. In this study we have begun to characterize the *C. albicans* orthologue of Ycf1 using gene disruption mutants and wild-type controls in cadmium and mercuric chloride susceptibility testing. Green fluorescent protein (GFP) fusion proteins were utilized to determine the cellular localization of the ABC transporter in *C. albicans* yeast and hyphal forms. Additionally, we use quantitative reverse transcriptase PCR to investigate ABC transporter gene expression. Our results indicate that the Ycf1 orthologue in *C. albicans* localizes to the vacuolar membrane of the fungi and appears to be involved in detoxification of cadmium and mercury, similar to the *S. cerevisiae* transporter. The gene expressions analyses revealed upregulation of the *YCF1* mRNA during mercury exposure, further confirming the transporter's role in mercury detoxification. Future studies will focus on the role of Ycf1 in vacuolar function and inheritance of *C. albicans* as well as its involvement in virulence of this opportunistic pathogen.

TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION

The eukaryotic microorganisms belonging to the Kingdom Fungi include molds and yeasts. Fungal saprobes are crucial for the decomposition of organic material, nutritional exchange and biogeochemical cycling in the environment. Humans have been using many species of fungi as food, for food production, or in recent decades in biotechnological applications such as production of enzymes, vitamins, and many other valuable chemicals. In scientific research, the yeast *Saccharomyces cerevisiae* has been an essential eukaryotic model organisms to study fundamental cell biological, genetic, and molecular biological processes. However, some fungi also have gained notoriety as mainly opportunistic pathogens in the increasing population of immunocompromised patients.

In the past, scientists considered fungi as closely related to plants; however, cell biological and molecular evidences have shown significant differences between these two groups of organisms. For example, chitin in the fungal cell wall is substituted by cellulose in plant cell walls. Additionally, as the principal decomposers in ecological systems, fungi do not undergo photosynthesis, but as chemoheterotrophs they gain energy through the break-down of organic material from the environment. While fungi are widespread in ecosystems, most of them are indiscernible due to their microscopic size, with the

exception of the fruiting bodies (mushrooms) of macroscopic fungi.

Fungi can have symbiotic, commensal, or parasitic relationships with animals, plants or other microorganisms. Some of species produce mycotoxins which can be highly toxic to human and animals. The members of the fungal kingdom can vary substantially in ecologies, life cycles, and morphologies, each contributing to the vast diversity of this organismal group. To date, our knowledge of fungi still is limited, only 5% of species have been identified and classified from an estimated 2.5 million to 4 million species in the world. Through the development of molecular techniques in recent decades, DNA sequencing has replaced the classical approach towards fungal classification by morphological characteristics. Some of the well-known species such as *S. cerevisiae* and *Candida albicans* were the first fungal species with available whole genome sequences [1].

Candida albicans is one of the most-studied fungal pathogens. It is a strictly diploid opportunistic pathogen that causes a wide range of infections in immunocompromised hosts. Most incidences of candidiasis are endogenous infections because the fungi are part of the normal human microbiota found in the mouth, gut, vagina, and skin in many individuals [2]. *Candida* species are mesophilic and thus able to grow at human body temperature, an important prerequisite for a pathogen – many other fungi are not pathogenic for warm-blooded animals because of growth temperature restrictions.

As a commensal in health individuals, *C. albicans* presents predominantly in its budding yeast form, usually in low numbers as it is only a minor component of the human microbiota. However, when normal physical, microbiological, and immune defenses are

interrupted or repressed because of trauma, broad-spectrum antibiotic use or immunosuppression, *C. albicans* and related non-albicans *Candida* species (e.g., *C. tropicalis, C. glabrata, C. krusei*) can become pathogenic and break through tissue barriers to cause superficial to deep-seated infections. The symptoms of candidiasis largely depend on the infecting *Candida* species, the location and severity of infection, and the host's immune status. In the mouth, candidiasis may present as oral thrush or pseudomembranous candidiasis. Vulvovaginal candidiasis may cause itching and burning, in some patients with certain immune defects with an increased rate of recurrence. Severe infections with invasion of blood vessels and hematogenous spread are seen especially in neutropenic patients, for example, those receiving chemotherapy for treatment of leukemia [3]. Most forms of severe candidiasis are associated with invasion and overgrowth in target organs which requires adaptation of the pathogen to different microenvironmental conditions. The ability to grow in different morphologies, as budding yeast or in filamentous forms (hyphae, pseudohyphae) helps *C. albicans* to adapt to different conditions in the hosts [4]. While a recent study claims that the yeast to hyphae transition is not always necessary for virulence of *C. albicans*, the hyphal form of *C. albicans* could still support infection, for example, by promoting invasion and hematogenous dissemination of the fungi [5]. In addition to morphological conversions, other adaptive survival mechanisms are involved in the pathogenesis of the different forms of candidiasis. Furthermore, development of antifungal drug resistance can exacerbate these diseases by rendering them difficult to treat successfully.

The aim of this study was to investigate the role of specific transport proteins in the detoxification of harmful substances and homeostasis of cellular functions. Among the

many transport proteins encoded in the *Candida* genome are energy-driven ATP-binding cassette (ABC) transporters. A specific subset of these transporters is not localized in the plasma membrane, but appears to perform transport functions in a specific organelle related to lysosomes in higher organisms, the fungal vacuole. Some vacuolar ABC transporters have been characterized in greater detail in the non-pathogenic *S. cerevisiae* and a smaller number in the opportunistic pathogen *C. albicans.* The latter, for example, harbors the MRP-related transporter Mlt1 which was discovered previously by Theiss et al. [6] and has been studied in greater detail [6, 7]. Here, we focus on the closely related transporter CaYcf1 whose putative orthologue has been characterized in *S. cerevisiae* as a vacuolar transporter involved in detoxification of cadmium; however, little is known about the functions of this transporter in the opportunistic pathogen *C. albicans.* This study is an initial *in vitro* functional characterization of this transporter which could be an essential step towards future *in vivo* studies in experimental animal models.

CHAPTER II

REVIEW OF LITERATURE

Candida albicans

C. albicans is a budding yeast with round or oval-shaped cells with diameters from 3 to 6 μm. Even though it is a diploid organism, no evidence of meiosis and a natural haploid phase has been found [8-10]. While budding is the mode of reproduction in the yeast form, *C. albicans* filamentous morphologies encompass the formation of pseudohyphae and true hyphae.

C. albicans is the most prevailing of the opportunistic *Candida* species – it causes the majority of clinical representations of candidiasis [11]. Whether or not disease occurs after invading the human body depends on the immune status of the human host, the number of invasive cells and expression of certain virulence factors. In the healthy, non-immunocompromised host, *Candida* is a commensal present in low numbers of predominantly yeast cells. However, in immunodeficient populations, *C. albicans* can transform its morphology from yeast to hyphae and invade mucosal tissue [12]. This pleomorphic ability of *C. albicans,* i.e., growing in the yeast, pseudohyphal, or hyphal form, is an important factor in the fungus' virulence. During the morphological switching from yeast to hyphae, additional virulence factors for adhesion and invasion are differentially expressed. Earlier studies have shown that different temperature and the

presence of carbon dioxide may affect the morphological forms of *C. albicans* [13]. The purpose for this morphological transitions appears to be adaptation to changing (micro)environmental conditions. Previous research showed that the virulence of *C. albicans* mutants unable to form hyphae (because of specific mutations in regulatory factors), is largely reduced [14]. Researchers argued that true hyphae are not essential for tissue invasion, because other species such as *C. glabrata* and *C. parapsilosis*, which are also relatively common causes of disseminated candidiasis, do not produce true hyphae [15]. *C. glabrata,* which only presents in yeast form, does have the ability to cause oropharyngeal candidiasis by binding to co-infecting *C. albicans* hyphae [16]. Generally, there appears to be no doubt that the ability to form hyphae increases virulence. For example, a recent study on *C. albicans* and *C. dubliniensis* in mouse systemic infection models showed that both species can produce hyphae and their virulence is potentiated by hyphae formation [17].

One way hyphae increase virulence is by induction of the expression of many additional proteins such as the agglutinin-like sequence protein 3 (Als3), which is important for adhesion, the secreted aspartic proteases family (e.g., Sap4, Sap5, Sap6, Sap9, Sap10), and the hypha-associated GPI-linked protein (Hwp1), another important adhesin [18]. Additionally, Hwp1 and Als3 as complementary adhesins could also aid in biofilm formation [19]. Besides its ability to improve adhesion, Als3 also plays an important role in invasion [20].

Overall, the current thinking is that hyphae break the normal cell defense boundaries and penetrate to deeper cell layers which causes damage in underlying tissues. According to

recent studies, the hyphal form will induce endocytosis or active penetration to invade host cells [21].

Apart from adhesion and invasion, another significant virulence factor of *C. albicans* is the ability to generate biofilms. The formation of biofilms is a continuous process, beginning with the attachment of yeast cells to their substrate, followed by formation of hyphae and extracellular material and leading to redistribution of cells from those mature glutinous complexes [22]. Furthermore, within biofilms, cells are more resistant to antibiotics and host immune responses [23].

Innate immune responses towards *C. albicans* are mediated by pattern recognition receptors (PRRs) which recognize the pathogen-associated molecular patterns (PAMPs) exposed by *C. albicans* cell walls. This process could help the immune system to differentiate harmful pathogens from normal microbiota [24]. Once *C. albicans* leaves the bloodstream, neutrophils and macrophages will stand guard to kill the intruder. Macrophages will simply try to engulf this pathogen and destroy the fungi intracellularly. However, neutrophils are the most effective and essential defending cells for protecting the human body from *C. albicans* infection with the ability to prevent hyphae formation [25]. Once a pathogen has been recognized, neutrophils will release reactive oxygen species (ROS), which induce oxidative stress to suppress fungal cell growth, and form neutrophil extracellular traps (NETs) [26]. NETs appear to be able to entrap and kill filamentous forms of *C. albicans* which cannot be successfully internalized by phagocytic cells. However, NET formation can also cause potential damage to surrounding tissue [26].

When *C. albicans* becomes a pathogen, dendritic cells as antigen presenting cells (APC) play an important role both in innate and adaptive immunity. By recognizing pathogen-associated molecular patterns on the *C. albicans* cell wall, dendritic cells could trigger the adaptive T cell immune protection against *C. albicans* infection [27]. The dynamic and complicated signaling to initiate adaptive immunity has not been fully understood.

In previous studies, *C. albicans* has been proven to have frequent karyotypic variation in response to different environmental stressors. Such variations in genome structure could pose a problem for many other fungi which regularly process through a post-meiotic haploid phase [28]. The diploid *C. albicans*, however, has a large capacity to tolerate karyotypic variation due to its highly heterogeneous and plastic genome [29]. In other words, *C .albicans* can adjust its genome in response to external triggers and quickly adapt to a changing environments.

Candidiasis

Candidiasis is the most common fungal disease. It can present as an acute, subacute, or chronic infection. Besides superficial and cutaneous mycoses, *Candida* spp. can cause deep-seated, hematogenously disseminated and systemic infections that are associated with high morbidity and mortality [30]. The clinical symptoms are commensurate with the area infected. In recent years, with the increased use of broad-spectrum antibiotics, hormones, and immunosuppressive agents, the incidence of candidiasis has increased while incidences of HIV-related opportunistic *Candida* infections have decreased in countries with available highly active antiretroviral therapy (HAART) [31].

Depending on the site of infection, candidiasis could be classified into different categories. For example, cutaneous candidiasis is more common in people with chronic exposure to wet environments who have interdigital erosions on the hands. A rash with itchy or painful feeling is the most common symptom, and peripheral areas are often scattered with red papules or pustules. *Candida* paronychia is common around infected nails [30-35].

Mucosal candidiasis refers to infections in genital areas or the mouth. Thrush is common in infants and young children. There is a clear white pseudomembrane at the oral mucous membranes, pharynx, tongue and gums with peripheral areas of redness. Genital candidiasis includes vulvovaginitis and balanoposthitis. Infections in males are rare, but vulvovaginal candidiasis is common in females of reproductive age. Vaginal secretions and grayish white patches on the vaginal wall with itching or burning are common symptoms of this infection [30, 31, 34-36].

Systemic *Candida* infections can affect all internal organs – relatively common are intestinal candidiasis and pulmonary candidiasis. These diseases are more common in clinically immunodeficient patients, especially in patients with long-term use of antibiotics or corticosteroids. Candidal enteritis is associated with abdominal discomfort, hypermotility, chronic diarrhea and anal itching. Candidal bronchitis is associated with cough and the presence of the pathogen in mucous phlegm. In addition, *Candida* can cause urinary tract inflammation, nephritis, endocarditis, meningitis, and even septicemia [30, 35].

Candida rashes are skin allergies caused by contact to the microorganism and its metabolic products, the main damage is formation of aseptic blisters, mostly found on fingers with psoriasis, rose blisters, seborrheic dermatitis and urticaria. Candidal endophthalmitis is seen in immunocompromised, diabetic, and candidemic patients. Although candidal endophthalmitis is a rare disease among ophthalmic infectious diseases, it has shown a tendency to increase with the widespread use of immunosuppressant therapy, long-term antibiotic therapy, long-term indwelling catheters, and intravenous injections [30, 34, 35].

Direct microscopic examination of specimens is an effective method to diagnose some forms of candidiasis. Culture of clinical isolates on specialized agar plates (e.g. CHROM agar) is another possible diagnostic approach for various *Candida* species. Germ-tube tests and biochemical tests may be the alternatives, but molecular methods have become increasingly relevant in *Candida* diagnostics [34].

Fungal Vacuoles

Vacuoles are ubiquitous structures in plant cells [37]. They are metabolic libraries and act as regulators of the intracellular environment. Generally speaking, vacuoles are round shaped organelles surrounded by a membrane with an internal aqueous solution containing salts, sugars, and pigments. As an enclosure surrounded by membrane, they could be employed for sequestration of toxic substances and waste products within the cell. Additionally, they also could aid in maintaining intracellular pH and provide storage capacity. The concentration of H^+ can reach very high levels and the intravacuolar pH is

generally acidic. The main functions of vacuoles in plants are the regulation of the osmotic pressure of cells, maintenance of the balance of water in cells as well as accumulation and storage of nutrients and various metabolites. Substances that are stored and accumulated in vacuoles include sugars, proteins, phospholipids, tannins, organic acids, plant alkaloids, pigments, and salts. The specific conditions vary depending on the type of plant, the site of organ tissue, and the degree of maturity. There are large amounts of small spherical vacuoles in juvenile cells at the plant root and stem tips. During cell maturation, multiple small vacuoles fuse into a large vacuole. A similar fusion process is commonly seen in fungi, although fungal vacuoles are more dynamic and single large vacuoles can readily separate into smaller vacuoles due to changes of environment. Fungal vacuoles, if mature enough, are able to reduce the requirement for cell nutrition. During the life cycle of fungal cells, vacuoles change in size, shape and number to response to needs of metabolic demands and cell cycle timing [38]. Several vacuolar functions are mediated by ABC transporters in the organelle's membrane.

The fungal vacuole also is responsible for degradation of intracellular components, similar to the function of lysosomes in mammalian cells [39]. Moreover, yeast vacuoles appear to play a role in autophagy [40]. In general, fungal vacuoles are extremely dynamic and frequently expanding and changing their numbers and shape in response to environmental change. Increasing evidence shows that during the life cycles of fungal pathogens, vacuoles are involved in morphological changes such as germ tube formation in *C. albicans* [41].

Another function for fungal vacuole is storage and sequestration of toxic compounds. Through specific transporters in the vacuolar membrane, lipids, amino acids, and

metabolic waste products can be pumped into vacuoles. Additionally, the ability to store glutathione-conjugation complexes is important for detoxification of toxic metals. While the exact mechanisms how these metals are detoxified with the help of vacuoles remain to be elucidated, specific ATP-binding cassette transporters appear to facilitate transport of these complexes across the vacuolar membrane.

ATP-Binding Cassette Transporters

The ATP-binding cassette (ABC) transporters are integral membrane proteins which have the capacity for directed, energy-driven transfer of chemicals and other components from one side of the membrane to the other via consumption of ATP.

These ubiquitous proteins are highly diverse and essential for many metabolism processes. While most ABC transporters have two trans-membrane domains (TMDs) and two nucleotide binding domains (NBDs), their functional diversity is also reflected by structural diversity.

Within the superfamily of ABC proteins, full-size ABC transporters share mutual topologies which comprise four domains, two TMDs with several transmembrane regions that help to establish the substrate translocation channel and two NBDs.

A detailed classification of ABC proteins depends on the sequence similarities of their nucleotide binding domains. Generally, ABC proteins are classified into seven subfamilies: the ABCA, ABCB, ABCC, ABCD, ABCE, ABCF and ABCG subfamilies [42]. However, the ABCE and ABCF subfamilies do not have the ability to transport

substrates across membranes because of lacking actual trans-membrane spans, nevertheless the presence of NBDs warrants their inclusion in the ABC protein superfamily [43].

As mentioned above, the core structure of ABC transporters are two TMDs consisting of several trans-membrane spans and interspersed amino acid loops as well as two NBDs which are highly conserved in all ABC transporters [44].

The NBDs belong to the subfamily of P-loop NTPases and have the function to hydrolyze ATP [45]. When binding and hydrolyzing ATP, NBDs will rearrange and cause conformational transformation of the TMDs [44]. The TMDs allow for substrate access to one or both sides of the membrane and are responsible for transport of the substrate via this transformation.

The ABC subfamily C (ABCC) transporters such as the human multiple drug resistance-associated protein 1 (Mrp1) or the yeast cadmium factor protein in *S. cerevisiae* (ScYcf1p) encompass a N-terminal extension with an additional TMD (TMD_0) [46].

Besides the TMD and NBD core structures, there are additional regulatory domains in ABC transporters called accessory domains. The regulatory functions include increasing the affinity to and enhancing the binding of substrates [47]. Functions not directly related to transportation such as supporting chaperoning and signal transduction are also mediated by these accessory domains [47].

The structural diversity of ABC transporters highly implies different transportation mechanisms, but even in transporters with similar structures, the transported range of substrates may be quite different [48].

Due to their wide range of locations, from intracellular organelle membranes, such as vacuolar or peroxisomal membranes, to the plasma membrane, ABC transporters provide a wide array of crucial functions. Additionally, ABC proteins also have important functions as ion channels, channel regulators and proteases [49]. Although each ABC transporter transports a limited range of substrates, the protein family has members capable of transporting ions, amino acids, nucleotides, polysaccharides, polypeptides, and even proteins. Some ABC transporters also catalyze the turnover of lipid bilayer phospholipids between the two layers (flippase activity), which is of great significance in homeostasis and functional maintenance of the membrane. ABC transporters are also involved in the resistance of pathogens to antimicrobial drugs such as clinically used antibacterial or antifungal drugs (see below for further details). In humans, the overexpression of multiple drug resistant (MDR1) and multiple drug resistant-like (MRP1) protein enhances resistance of tumor cells to chemotherapeutic drugs [50].

ABC transporters are also involved in essential transport functions for maintaining the blood-brain barrier to keep toxin and metabolic waste products from entering the CNS [51]. Inherited diseases like cystic fibrosis are caused by loss of function mutations in the CFTR ABC transporter [52]. Overall, better understanding of the cellular functions and mechanisms of transport by ABC transporters is important for cell biology, chemotherapy and prevention of human diseases. As eukaryotic microorganisms amenable to genetic manipulation, fungi such as *S. cerevisiae* and *C. albicans* have become ideal models for investigating the functional roles of ABC transporters.

Research in *C. albicans*, with some exceptions in infection-related studies, has benefited considerably from research in the model microorganism *S. cerevisiae*. *C. albicans*

naturally shares some properties with *S. cerevisiae*, including most of the inventory of the ATP Binding Cassette (ABC) transporters in the vacuolar membrane. For example, putative orthologues of the *S. cerevisiae* ABCC transporters yeast cadmium factor 1 (ScYcf1p) and bile pigment transporter 1 (ScBpt1) appear to be encoded in the *C. albicans* genome [53, 54].

This study focused on the *C. albicans* orthologue of ScYcf1p due to its prolific functions in *S. cerevisiae*. Ycf1p is the best characterized member of the ABCC subfamily of ABC transporters in *S. cerevisiae.*

Ycf1 Protein

Similar to other full-size ABC transporters, ScYcf1p has two NBDs and two TMDs. Additionally, the protein harbors an extra N-terminal extension (NTE) with five trans-membrane spans and an additional cytosolic loop (L_0), typical features of full-length ABCC subfamily transporters[46]. It has been demonstrated that the short cytosolic loop is essential for substrate specificity, which means it may be involved in binding of glutathione-conjugate complexes, whereas the N-terminal extension is involved in vacuolar trafficking [55].

The yeast cadmium factor 1 protein in *S. cerevisiae* (ScYcf1p) was first found when the yeast was exposed to cadmium sulfate solution – overexpression of the gene conferred cadmium resistance [56-59]. Conversely, inactivation of the *ScYCF1* gene rendered *S. cerevisiae* hypersusceptible to cadmium. Interestingly, while ScYcf1p was first believed to reside in the plasma membrane, it actually resides in the vacuolar membrane [57].

Previous studies have proven that Ycf1p can pump cadmium into the vacuole via bis(glutathionato)cadmium complexes ($Cd•GS_2$) and trapping of these complexes inside the vacuole clearly demonstrated the heavy metal detoxification capacity of ScYcf1p [56]. Another study also showed that ScYcf1p plays a role in mercury detoxification [60]. Similar to cadmium detoxification, glutathione-conjugated mercury will be firstly formed and then sequestered into the yeast vacuole by ScYcf1p [60]. This indicates not only cadmium, but other heavy metals undergo similar detoxification mechanisms mediated by ScYcf1p.

Interestingly, ScYcf1p exhibits high amino acid similarity (63%) with the human multidrug resistance-associated protein 1 (Mrp1p) [57, 61]. Therefore, it is possible is that these proteins share similar functions. A comparative study revealed that heterologous expression of human MRP1p complemented the disruption of the *ScYCF1* gene by restoring the glutathione-conjugate transporter function in these yeast mutants [61]. This experiment suggested that ABCC transporters may share similar functions even across kingdom boundaries, making yeast a promising research model for investigation of these transporters.

In addition to its major function in metal-glutathione-conjugate transport ScYcf1p also appears to be involved in the regulation of homotypic vacuolar fusions. ScYcf1p plays a role in recruitment of the Soluble N-ethylmaleimide-sensitive factor Attachment protein REceptor (SNARE) vacuolar morphogenesis protein 7 (Vam7p) for stimulation of vacuole fusions [62]. The ATPase function of ScYcf1p was required for this fusion reaction to take place.

ScYcf1p also plays an important role in maintaining cellular balance under high-salt stress via its glutathione recycling function. If the expression of *ScYCF1* is impeded, superoxide dismutase proteins (Sod1, Sod2) will compensate and maintain resistance to salt stress [63].

A recent study has shown that the copper chaperone for superoxide dismutase 1 (Ccs1) protein may be involved in ScYcf1p-mediating cadmium resistance because *CCS1* deletion blocks maturation of Sod1 and leads to oxidative inactivation of ScYcf1p [64]. Thus, copper metabolism, antioxidant enzymes, and cadmium defense appear to be intertwined.

In contrast to the accumulating information about the functional roles of ScYcf1p in *S. cerevisiae*, little known about the putative orthologue CaYcf1p in *C. albicans*.

Mlt1 Protein

Multidrug resistant protein-like transporter (Mlt1) is an ABC transporter in *C. albicans* which is localized in the vacuolar membrane. It was first isolated in 2002 and *MLT1* gene deletion mutants helped to reveal the protein's involvement in virulence of *C. albicans*.[6]

Like ScYcf1p, Mlt1p belongs to ABCC subfamily of ABC proteins. The structure of Mlt1p with the usual two TMDs and NBDs of the full-length ABCC transporters, is similar to ScYcf1p, also including the additional N-terminal extension domain with five trans-membrane helices, but substantial differences in the protein sequences [6, 7].

The gene designation *MLT1* was given because of the structural similarities of the encoded protein with the human multidrug resistance-associated protein Mrp1; however, overall the protein sequence is more similar to ScYcf1 than Mrp1 [6]. Consequently, Mlt1p has been misnamed by some researchers as the *C. albicans* orthologue of ScYcf1p. However, the existence of a *CaYCF1* gene and emerging evidence of distinctive functions of Mlt1p do support the notion that Mlt1p is not orthologous to ScYcf1p. For example, Mlt1p has been proven to be involved in endocytosis, oxidoreductase activities in cells, biofilm formation, hyphae formation, and virulence of *C. albicans* [7]. Mlt1p-defective mutants failed to invade liver parenchyma of mice during peritoneal infection [6]. With the deletion of both *MLT1* alleles in *C. albicans,* mutant cells are more susceptible to H_2O_2 and associated reactive oxygen species (ROS) [7]. However, ROS will also increase the expression level of glutathione in cells, which could overlap with the major function of ScYcf1p as glutathione-conjugation complex transporter [57].

Thus, there is high possibility that Mlt1p is functionally closely related to ScYcf1p or its putative homologue CaYcf1p in *C. albicans*, not only because of highly similar protein structures, but also because of the involvement of both transporters in the response to oxidative stress. Investigation of functional overlaps between Mlt1p and CaYcf1p was one of the goals in this research project.

Antifungal Drug Resistance

Antifungal drug resistance is based on several major modes of resistance. One is natural or acquired resistance associated with reduced binding of a drug to its target due to

binding site modifications. Increased expression of the drug target could also lead to resistance development. Additionally, the action of efflux pumps which expel drugs from the intracellular environment and thus reduce or preclude contact with the drug target. Clinical resistance in combination with impaired immune functions or other co-infecting pathogens can render some fungal infections incurable by standard therapeutic approaches [65]. While fluconazole has been an effective treatment for several forms of candidiasis, resistance of *C. albicans* strains to this azole drug has increased substantially in recent years [66, 67].

Azoles are the most widely used antifungal drugs since 1958 [68]. The first azole antifungal drug was ketoconazole for systemic infections and later it was replaced fluconazole and itraconazole [69]. The antifungal mechanism of azoles is inhibition of the cytochrome P450 enzyme lanosterol 14α-demethylase which is a key enzyme in the biosynthesis of ergosterol, a crucial component of the fungal plasma membrane [70]. Blockage of ergosterol synthesis by azole interferes with fungal growth, but does not necessarily lead to cell death, i.e. the drugs are only fungistatic.

Efflux pumps conferring resistance to azoles are located in the plasma membrane. The *Candida* drug resistance 1 and 2 (Cdr1, Cdr2) proteins are transporters belonging to ABCG or Pleiotropic Drug Resistance-like subfamily of the ABC protein superfamily [71] – they are the major efflux pumps reducing the intracellular drug concentration [72]. Cdr2p appears to exert supportive functions for Cdr1p. *CDR2* expression alone is inefficient to reduce drug concentrations, while exclusive *CDR1* expression is sufficient. In contrast to the Cdr proteins, the multidrug resistance protein 1 (Mdr1) is a member of

the major facilitator superfamily of transporters and seems restricted to the efflux of fluconazole [73].

The initial goal of this project was to determine the location of CaYcf1p in *Candida albicans*. I used green fluorescent protein fusion (GFP) protein constructs to indicate the potential location for CaYcf1p and compare it to previously published Mlt1p localization results [6]. For functional characterization, the susceptibilities of *CaYCF1* mutants to cadmium and mercuric chloride and antifungals were tested in comparative analyses with *MLT1* mutants. Real-time reverse transcriptase PCR was utilized for *CaYCF1* and *MLT1* gene expression analysis during exposure to toxic metals.

CHAPTER III

METHODOLOGY

Fungal Strains and Culture Conditions

C. albicans strains were routinely grown in YPD (yeast extract 10g/l, Peptone 20g/l, Dextrose 20g/l). Frozen stocks were kept at -80℃ in YPD plus 20% glycerol as cryopreservative. Additional media such as synthetic defined medium (SD; yeast nitrogen base + complete Supplement Mixture (CSM) + 20% Glucose; Sunrise Science Products, San Diego, CA) and the cell culture media FluoroBrite DMEM (Dulbecco's Modified Eagle Medium; Gibco, Thermo Fisher Scientific) and RPMI 1640 (with glutamine, without bicarbonate; Gibco, Thermo Fisher Scientific) were used as indicated in the following sections. Table 1 shows the *C. albicans* wild-type and mutant strains used in this study. Individual strain descriptions are summarized here:

C. albicans SC5314 (CA200) is the wild-type parent strain used as control.

C4GFP (CA201) and 6478GFP-1 (CA306) are mutant strains expressing C-terminal fusion proteins of GFP with Mlt1p and Ycf1p, respectively. These are Ura^+ strains derived from the ura^- strain *C. albicans* CAI4 [74].

Strain ST13-63 (CA239) is a complete *MLT1* knock-out mutant with two disrupted alleles of the *MLT1* gene.

Strain ST13-K1 (CA241) is a *MLT1*-complemented mutant derived from strain ST13-63 which contains one functional *MLT1* allele.

Strain 353M1F34 (CA398) is a complete *YCF1* knock-out mutant with two disrupted alleles of the *YCF1* gene.

Strain 398YCFF1 (CA437) is an *YCF1* complemented mutant derived from strain 353M1F34 which contains one functional *YCF1* allele.

Strain S49F6 (CA394) is a *MLT1* and *YCF1* double knock-out mutant with both alleles of the *MLT1* and *YCF1* genes disrupted.

Strain S49F6R3FF35 (CA396) is an *YCF1*-complemented mutant derived from strain S49F6 with one intact allele of *YCF1*.

Strain CAG31 (CA202) is a mutant strain expressing cytosolic GFP under the control of the constitutive β-Actin (*ACT1*) gene promoter.

Growing *Candida albicans* mutants from stocks

All wild-type and mutant *C. albicans* strains were inoculated on YPD agar plates from -80℃ stocks and grown for two days at 30℃. Single colonies were used to inoculate liquid cultures in YPD broth or other media. Depending on the experimental conditions, liquid cultures were incubated at 25℃, 30℃ or 37℃ with or without shaking at 180rpm on a rotatory shaker.

Table 1. *C. albicans* wild-type and mutant strains used in this study

Strain	Parent	Genotype	Strain ID	Source / Reference
SC5314		wild-type strain	CA200	[75]
C4GFP	CAI4	*MLT1/MLT1-GFP-URA3*	CA201	[6]
6478GFP-1	CAI4	*YCF1/YCF1-GFP-URA3*	CA306	Koehler, G., unpublished
ST13-63	ST13-12	*mlt1-1Δ::hisG/mlt1-2Δ::hisG*	CA239 (*mlt1/mlt1*)	[6]
ST13-K1	ST13-63	*MLT1-MPA^R /mlt1-2Δ::hisG*	CA241 (*MLT1/mlt1*)	[6]
353M1F34	353MI	*ycf1Δ::FRT/ycf1Δ::FRT*	CA398 (*ycf1/ycf1*)	Koehler, G., unpublished
398YCFF1	398YCF1	*ycf1Δ::FRT/YCF1-FRT*	CA437 (*YCF1/ycf1*)	Koehler, G., unpublished
S49F6	S49	*mlt1-1Δ::hisG/mlt1-2Δ::hisG ycf1Δ::FRT/ycf1Δ::FRT*	CA394 (*mlt1/mlt1 ycf1/ycf1*)	Koehler, G., unpublished
S49F6R3FF35	S49F6R3	*mlt1-1Δ::hisG/mlt1-2Δ::hisG ycf1Δ::FRT/YCF1-FRT*	CA396 (*mlt1/mlt1 ycf1/YCF1*)	Koehler, G., unpublished
CAG31	CAI4	*ACT1/P$_{ACT1}$-GFP-URA3*	CA202	[76]

Confocal Microscopy of GFP-labeled *Candida albicans* Mutants

The *C. albicans* expressing GFP fusion proteins of Mlt1 or Ycf1 were used for protein localization experiments. The cytosolic GFP expressing strain CAG31 was used as a control. In addition to GFP fluorescence, strains were stained with additional dye for visualization of cellular structures such as the fungal cell wall or nuclei.

Hoechst 33342 staining

Hoechst 33342 (bisBenzimide H 33342 trihydrochloride; Millipore Sigma, Cat. No. 14533, St Louis, MO) was used as nuclear counterstain (with incubation up to 60 minutes). Stocks were prepared at 10mg/ml and stored at -20 ℃.

Calcofluor White staining

Calcofluor White (aka Fluorescent Brightener 28; MP Biomedicals, Santa Ana, CA), can be used to visualize the fungal cell walls. The stain was used at a final concentration of 10 μg/ml and staining for 30 – 60 minutes. Calcofluor White staining can be visualized by 405nm excitation and DAPI emission settings.

Confocal Microscopy

C. albicans strains expressing GFP were grown in liquid YPD cultures and then used to inoculate 300 μl Gibco FluoroBrite DMEM medium (DMEM without phenol red) or SD

medium in eight-chamber culture slides. To determine whether the different forms of *C. albicans* would affect the location of Ycf1p, we incubated these cells under 30 ℃, 37 ℃ and 37 ℃ and 5% CO_2 in a cell culture incubator for two to five hours. Subsequently, cells were stained with Hoechst 33342 by removing the medium and replacing it with fresh medium containing the Hoechst 33342 dye diluted to 0.1mg/ml and incubating the slides for 15 to 60 minutes to achieve staining of nuclei. Concomitantly, in several experiments cells additionally were stained with Calcofluor White as described above.

Before observing under the confocal microscope, media in all chambers were removed and cells were washed with fresh media. After removal of the final wash, 200 µl of a 0.7% agarose solution in the respective medium were added to cover and immobilize cells in the chambers. Hoechst 33342 and/or Calcofluor White staining was detected using a Leica SPE confocal laser scanning microscope (Leica Microsystems Inc., Buffalo Grove, Il) with a 405nm laser for excitation and DAPI emission settings. GFP fluorescence was detected using excitation with 488nm laser and Leica emission settings for enhanced GFP (emission maximum at 510nm).

Spot Assay of *Candida albicans* ABC Transporter Mutants

100 µg/ml cadmium chloride YPD agar plates (60 ml in 150 x 15 mm petri dishes) were prepared for the spot assay. For inoculation, overnight cultures in YPD at 30 ℃ of the wild-type strain CA200 and the ABC transporter mutant strains CA239, CA241, CA394, CA396, CA398, and CA437 (see Table 1) were prepared. The inocula were adjusted to equal optical densities of OD_{600nm} = 1.0 in YPD broth. Subsequently, a dilution series was

prepared for each strain from the starting OD_{600nm} = 1.0 by 1:5, 1:25, 1:125, 1:625, and 1:3125 dilutions. For each strain, 2 µl of each dilution were spotted in a row on the cadmium chloride YPD plates. The spot assays were prepared for incubation at 30 ℃ and 37 ℃. Plates were photographed after 4 days of incubation at the respective temperature. Overall, experiments were performed in triplicates.

Disk Diffusion Assays with Cadmium, Mercury, and Lead Compounds

The CA200 wild-type and the CA239, CA241, CA394, CA396, CA398, CA437 mutants were grown overnight in YPD broth. The OD_{600nm} of each culture was measured and each strain was diluted to an OD_{600nm} of 0.01 in 25 ml liquid YPD soft agar with 0.7% w/v agarose which was kept at 45 ℃ to avoid premature solidification. The inoculated soft agars were poured immediately on top of solidified 60 ml YPD agar in 150 x 15 mm petri dishes and allowed to solidify at room temperature. For each tested compound, five paper disks were carefully loaded with 15 µl of 50 mg/ml, 5 mg/ml, 500 µg/ml, 50 µg/ml and 5 µg/ml of either mercuric chloride, cadmium chloride, or lead-acetate solution. Each set of disks was deposited on the solidified YPD soft agar overlay plates. The plates were incubated at 30 ℃ for 2 days and subsequently photographed to document growth inhibition zones around the disks.

Broth Microdilution Assays for $CdCl_2$ and $HgCl_2$ in ABC Transporter Mutants

Various media were tested for the broth microdilution assays with cadmium and mercury salts. Following these pilot experiments, the media of choice for the assays were SD medium and RPMI 1640 buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) and 2% glucose (RPMI-1640-MOPS-2%Glc), a medium adapted from NCCLS recommendations for *C. albicans* antifungal drug susceptibility testing [77].

For the microdilution assay, the CA200 wild-type strain and the ABC transporter mutant strains CA398, CA437, CA394, CA396, CA398, and CA437 were grown overnight in SD medium. The optical densities of the cultures were measured and the strains were diluted to an OD_{600nm} = 0.1 in SD medium. One hundred µl of 400 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml cadmium chloride or mercuric chloride solution in the same medium were added into 96-well flat-bottom microplates. Control wells with no addition of cadmium or mercury salts were included. Subsequently, 100 µl of the strain dilutions were added to each control well and the wells containing the toxic metal salt dilutions. This procedure resulted in final cadmium or mercuric chloride concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.125, and 0 µg/ml and an OD_{600nm} of 0.05 for each strain. The microplates were incubated at 37 ℃ without shaking. After 24 and 48 hours of incubation, plates were shaken on a microplate shaker for 5 mins at 800rpm and subsequently the optical densities at 600nm were measured in a Biotek Synergy 2 Multi-mode plate reader (BioTek, Winooski, VT). Experiments were repeated twice in duplicates.

Broth Microdilution Assay for Fluconazole in ABC Transporter Mutants

The susceptibility of *C. albicans* wild-type and control strains to fluconazole was tested using a broth microdilution assay similar to the assay described in the previous section, following NCCLS guidelines [77]. Briefly, fluconazole dilution series were prepared from an aqueous 12,800 mg/L fluconazole stock solution to achieve final concentrations of 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, and 0.125 mg/L in 96-well microplates. The growth medium of choice was RPMI-1640-MOPS-2%Glc (see above). Wells were inoculated with *C. albicans* strains grown in overnight cultures and adjusted to OD_{600nm} of 0.1 – following use of 100 µl cell suspension and 100 µl 2x drug solution the final starting OD_{600nm} was 0.05. Control wells included wells with no drug and wells with no inoculation of *C. albicans* cells as blanks. The assay microplates were incubated at 37 ℃ without shaking. After 24 and 48 hours of incubation, plates were shaken on a microplate shaker for 5 mins at 800rpm and subsequently OD_{600nm} values were measured in a Biotek Synergy 2 Multi-mode plate reader. Experiments were repeated twice in duplicates.

RT-qPCR of *CaYCF1* and *MLT1* mRNA Expression in Presence of $HgCl_2$

C. albicans cultures for RNA extraction

The wild-type *C. albicans* strain CA200 (genotype *MLT1/MLT1 YCF1/YCF1*), the complete *MLT1* gene disruption mutant CA239 (*mlt1/mlt1*) and its complemented derivative CA241 (*mlt1/MLT1*) as well as the *YCF1* gene disruption mutant CA398 (*ycf1/ycf1*) and the *YCF1*-complemented strain CA437 (*ycf1/YCF1*) were used for *MLT1*

and *YCF1* mRNA expression analyses by reverse transcriptase quantitative PCR (RT-qPCR).

The strains were cultured overnight in SD medium at 30 ℃ in a shaking incubator. The next morning, the optical densities of each culture were determined and the strains were diluted in fresh medium to an OD_{600nm} of 0.1. The resulting cultures were incubated in 6-well tissue culture plates (3 ml per well) at 30 ℃ with 115 rpm rotatory shaking. After 3 hours of growth, 0.9 µl 50 mg/ml $HgCl_2$ solution was added to the wells 15 µg/ml as the final $HgCl_2$ concentration. The control wells for each strain did not receive $HgCl_2$. Subsequently, plates were incubated under the same conditions for another 2 hours. For total RNA isolation, cultures were transferred to 15 ml tubes and cells were harvested by centrifugation (10 mins at 4,500 × g, 4 ℃) and immediately frozen at -80 ℃ until RNA extraction.

RNA extraction and quality control

Total RNA was isolated using the ZR Quick-RNA™ MiniPrep Plus kit (Zymo Research, Irvine, CA) according to the manufacturer's instructions. Briefly, the harvested *Candida* cells were directly homogenized using bead bashing tubes (Zymo Research) with 1X DNA/RNA Shield™ and 200 µl nuclease free water in a Mini-Beadbeater -96 (Biospec Products, Bartlesville, OK) with beating for two times 1 min. Subsequently, tubes were centrifuged for 1 min at 15,000 × g. and the supernatant was transferred to new nuclease-free tubes for RNA isolation. Depending on the amount of supernatant, an equal volume of RNA Lysis Buffer was added to each tube to complete lysis. To remove the majority of genomic DNA, the samples lysed in RNA Lysis Buffer were transferred into Spin-Away™-Filter and centrifuged at 15,000 g for 1 minute. Subsequently, equal volumes of

ethanol (100%) were added to the sample flow-through. Samples were well mixed by gentle pipetting up and down and transferred into a Zymo-Spin IIICG™ Column. The columns were centrifuged at 15,000 ×g for 30 seconds. Following centrifugation, the flow through was discarded and RNA samples were immediately subjected to in-column DNAse I treatment following the manufacturer's protocols. For this treatment, 400 µl RNA Wash Buffer were added to each Spin IIICG™ column and centrifuged at 15,000 ×g for 30 seconds. After the washing step, the columns were placed in new collection tubes and a DNase I solution mixture of 5 µl (1U/µl DNase I and 75 µl DNA digestion buffer) was directly added to each column matrix and incubated for 15 min at 25℃. Following DNase I treatment, 400 µl RNA Prep Buffer was added to each column and these were centrifuged at 15,000 ×g for 1 minute. For washing the RNA bound to the columns, 700 µl of RNA Wash Buffer were added to the columns, followed by and centrifugation at 15,000 ×g for 30 seconds. The washing step was repeated with 400 µl RNA wash buffer and centrifugation at 15,000 ×g for 2 minutes to ensure the complete removal of wash buffer. Finally, each column was placed in a sterile nuclease-free Eppendorf tube and 100 µl DNase/RNase free water were added. The RNAs were eluted by centrifugation at 10,000 ×g for 30 seconds. The eluted total RNAs were stored at -80℃.

RNA concentrations were determined by 260 nm and 280 nm readings using a Take 3 Micro –volume plate in a BioTek Synergy 2 Multi-mode plate reader. The integrity of the extracted RNAs was verified by agarose gel electrophoresis (1% agarose; TAE buffer). Following gel electrophoresis, gels were stained with SybrSafe (Thermo Fisher Scientific)

and imaged on a Typhoon 9410 Variable Mode imager. Clearly, defined 16S and 23S ribosomal RNA bands were indicative of good quality RNAs.

Reverse Transcription and quantitative PCR

Reverse transcription to generate cDNA was performed using Applied Biosystems High Capacity RNA-to-cDNA kit following manufacturer's instructions (Applied Biosystems Foster City, CA). Briefly, 10 µl of 2x RT Buffer, 1 µl of 20x RT enzyme, and up to 9 µl of total RNA sample (2 µg maximum capacity per 20 µl reaction) were pipetted into a 0.5 ml PCR tube. Nuclease-free water was added to reach a final volume of 20 µl. The Eppendorf tubes were placed on a BIO-RAD DNA Engine PT200 thermal cycler and incubated for 60 min at 37 ℃ followed by 5 min at 95 ℃ to stop the reaction and cooling to 4 ℃. The cDNA reaction tubes for each sample were stored at -20 ℃ for future use.

Quantitative PCR reaction mixtures were prepared using PowerUP™ SYBR Green Master Mix (Applied Biosystems Foster City, CA). Each reaction was run in duplicate in a final volume of 10µl containing 5 µl PowerUp SYBR Green Master Mix (2X), 0.2 µl each of the gene-specific forward and reverse primers, 1 µl of cDNA template, and 3.6 µl of nuclease-free water. Primer sequences for the *MLT1* and *CaYCF1* genes as well the *C. albicans* Actin 1 (*ACT1*) housekeeping gene are shown in Table 2. Reaction were set up in 96-well PCR semi-skirted reaction plates and sealed with ThermalSeal adhesive film (Phenix Research Products, Candler, NC).

The PCR reaction plates were centrifuged at 4000 g for 5 minutes at 4 ℃. Quantitative PCR reactions were run on an Applied Biosystems 7500 Real-time PCR system with the following reaction parameters: 2 min at 50 ℃, 2 min at 95 ℃, 40 cycles of denaturation

for 15 seconds at 95 ℃ and annealing/extension for 1 min at 60 ℃. Relative quantitation qPCR data were analyzed using the ΔΔCq method.

Table 2. Oligonucleotide primers used in the RT-qPCR

Primer	5'-Sequence-3'	Amplicon Length (nt)
ACT1_F	TGGTAGAACCACCGGTATTGTTTT	66
ACT1_R	GCGTAAATTGGAACAACGTGAGTAA	
WH_MLT1_F	AATCTCCCTGATGGGGACCA	150
WH_MLT1_R	ATGAGCGTCCACTGCTGAAA	
WH_YCF1_1	CCACCACAAGATTGGCCTCA	146
WH_YCF1_R	GCACCGGTTCTACCCACAAT	

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CHAPTER IV

FINDINGS

Functional characterization of the *C. albicans* ABC transporter CaYcf1 in this study encompassed detection of the putative cellular location of the transporter and toxic metal susceptibility assays with specific gene disruption mutants to define some of the transport functions of this protein. All experiments included comparative analyses with the related Mlt1 transporter. Furthermore, gene expression analyses were performed in the presence of mercuric chloride to assess whether the *CaYCF1* gene is induced under these conditions and whether any compensatory gene regulation takes place in *CaYCF1* and *MLT1* gene disruption mutants.

Cellular Localization of the CaYcf1 ABC transporter

Confocal microcopy was used to detect where the *YCF1::GFP* and *MLT1::GFP* fusion proteins localize in *C. albicans* cells. For comparison, strain CAG31 was used to demonstrate cytosolic expression of GFP. The following section shows representative images illustrating that the C-terminal GFP fusion proteins of both ABC transporters localize to the vacuolar membrane.

Figure 1 depicts images of the *MLT1-GFP* fusion protein in yeast and pseudohyphal cells of *C. albicans* C4GFP. The fusion protein clearly localizes to the membranes of large and multiple smaller vacuoles.

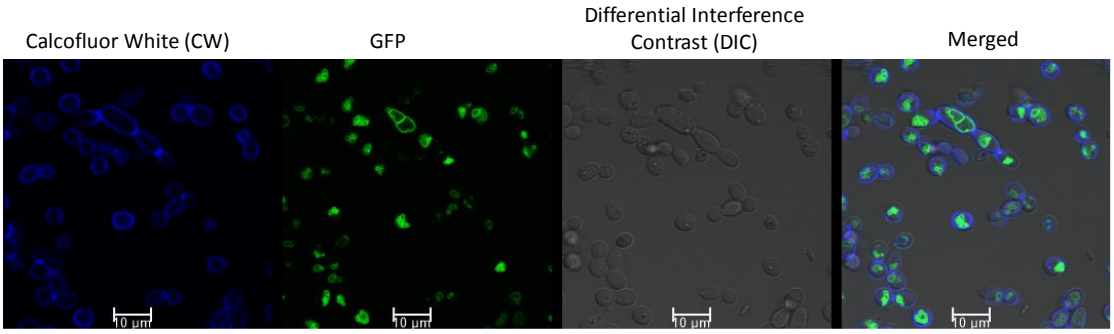


Fig. 1. Confocal imaging of strain C4GFP expressing the *MLT1::GFP* fusion protein

Calcofluor White (CW) cell wall staining and GFP fluorescence (GFP) confocal images as well as differential interference contrast imaging (DIC) using transmitted light detection are shown. The right panel (Merged) represents an overlay of the images to the left. The ABC transporter GFP fusion protein does not localize to the plasma membrane, but to the vacuolar membrane. Cells with vacuoles in different sizes and configurations are shown.

In Figure 2 C4GFP budding yeast cells and pseudohyphae are shown with GFP fluorescence in multiple vacuoles of different sizes and numbers per cell.

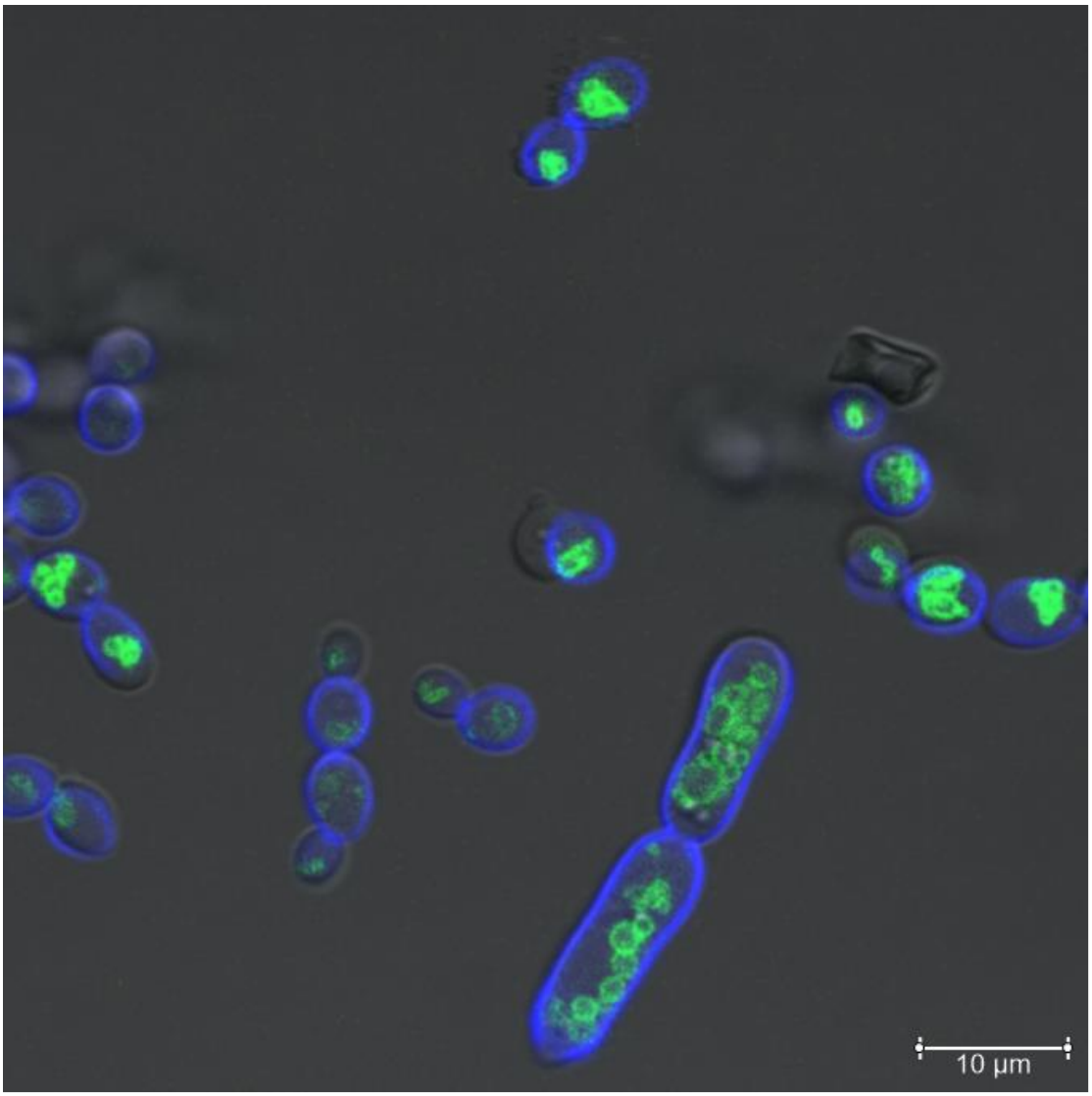


Fig. 2. Overlay image of strain C4GFP expressing the *MLT1::GFP* fusion protein

Confocal images of CW and GFP fluorescence were merged with the DIC image. The localization of GFP fluorescence clearly can be seen in the membranes of multiple small vacuoles in most cells that have not fused into a large vacuole. Some cells in the DIC image did not overlap completely with the fluorescent images because they had moved during imaging.

In contrast to the distinct localization of the Mlt1-GFP ABC transporter fusion protein in vacuolar membranes, the cytosolic GFP expressed by the constitutive *ACT1* promoter in strain CAG31 can be seen throughout most of the intracellular space (see Figure 3).

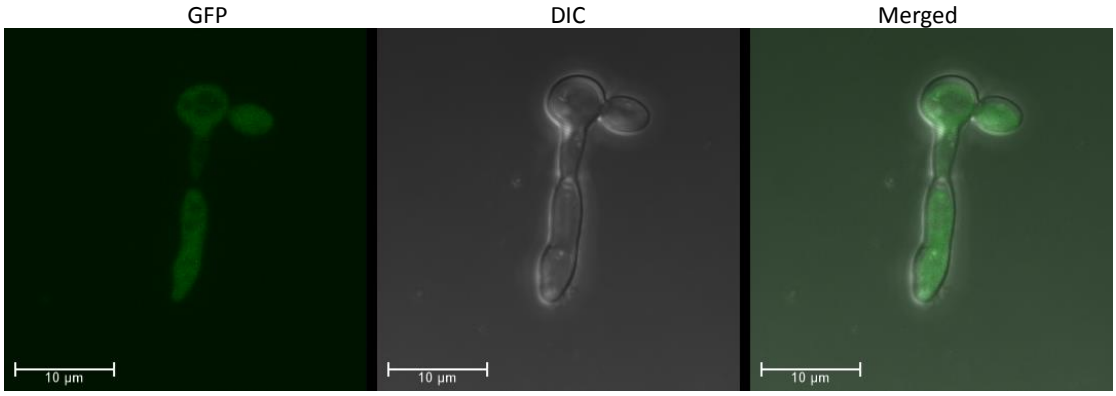


Fig. 3. Expression of cytosolic GFP in strain CAG31

For comparison with the ABC transporter fusion proteins, the images show a pseudohypha expression cytosolic GFP under control of the constitutive *ACT1* promoter. GFP fluorescence can be seen throughout the cytoplasm with the exception of areas occupied by vacuoles or nuclei.

In strain 6478GFP-1, the introduced *C. albicans YCF1::GFP* gene leads to expression of a fusion protein that, similar to the Mlt1-GFP protein, appears to localize to the vacuolar membrane (see Figure 4). Overall, the expression of this protein seems weaker than the expression of Mlt1-GFP in strain C4GFP.

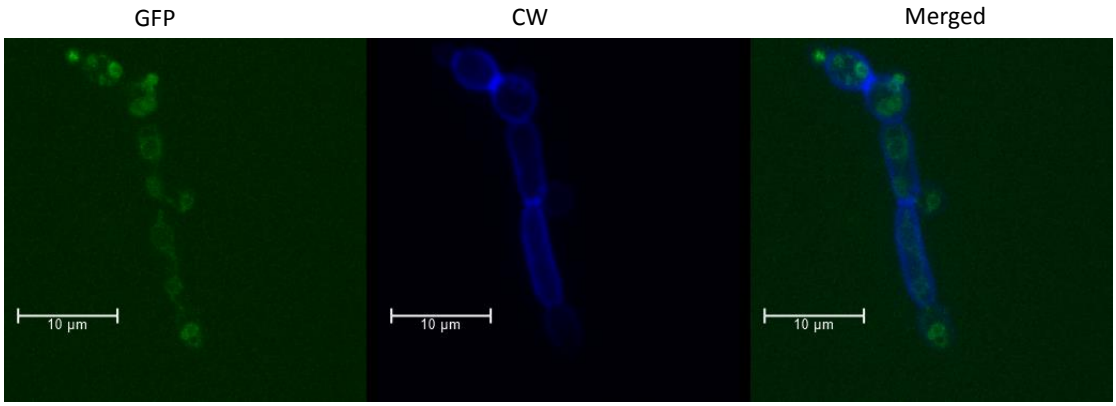


Fig. 4. Confocal imaging of strain 6478GFP expressing the *YCF1::GFP* fusion protein

GFP fluorescence (GFP) and Calcofluor White (CW) as well as the overlay of both (Merged) are shown. Similar to Mlt1-GFP, the ABC transporter GFP fusion protein does not localize to the plasma membrane, but to the vacuolar membrane. A pseudohypha with multiple smaller vacuoles is shown. Note the thin tubular connections between larger vacuoles in the germ tube.

C. albicans 6478GFP-1 germ tubes, precursors of true hyphae, also show green fluorescence in vacuolar membranes, indicating that this morphological form also expresses the Ycf1-GFP protein (Figures 5 and 6).

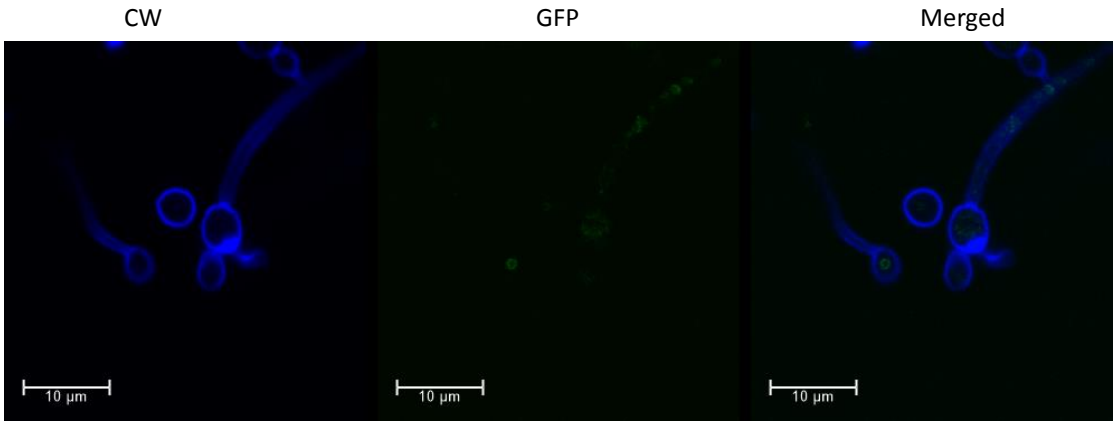


Fig. 5. Confocal imaging of the Ycf1-GFP protein in *C. albicans* germ tubes.

The green, ring-like fluorescence visible in the GFP and merged image indicates multiple vacuoles in mother cells and a germ tube.

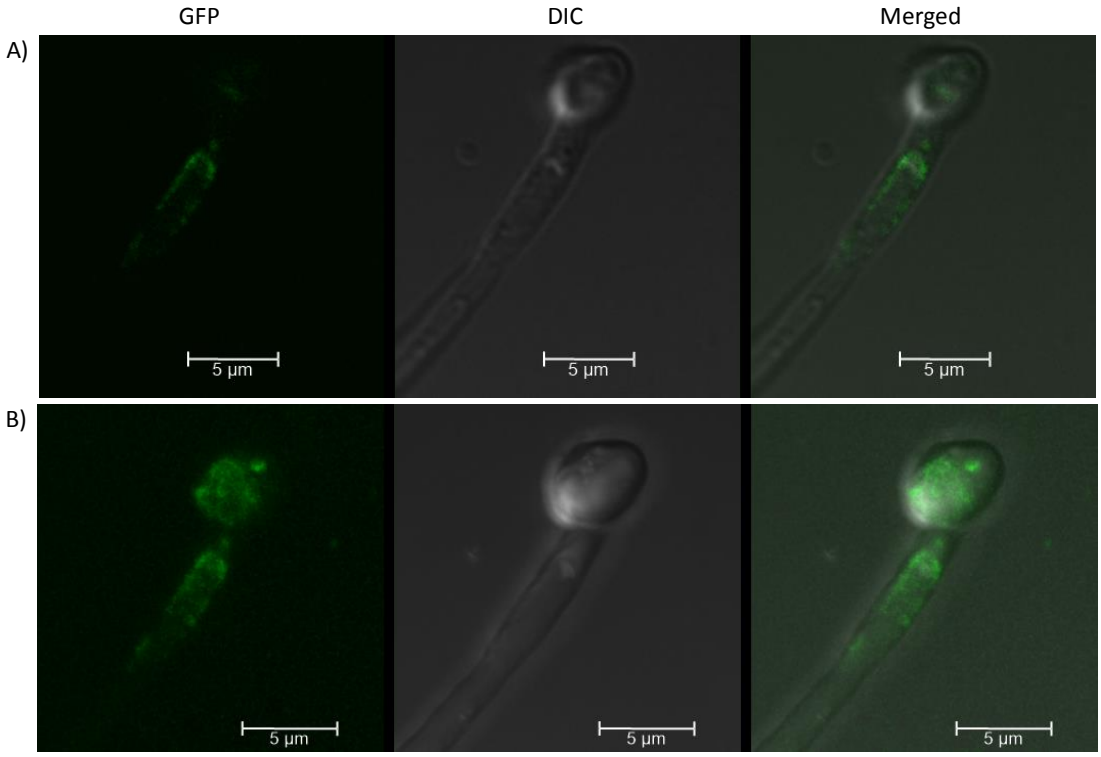


Fig. 6. Confocal imaging of the Ycf1-GFP protein in a single *C. albicans* germ tube and mother cell.

GFP fluorescence, DIC, and merged images are shown from a single Z-stack slice (A) and a maximum projection of 30 Z-stack slices. In the DIC image, a long vacuole can be seen in the germ tube which appears to accumulate the GFP fusion protein (see GFP image in A). The maximum projection GFP image in (B) reveals an agglomerate of smaller vacuoles in the mother cell.

Figure 7 shows another example of Mlt1-GFP fluorescence in budding yeast cells, within a single section of a confocal Z-stack and as maximum projection of the entire Z-stack.

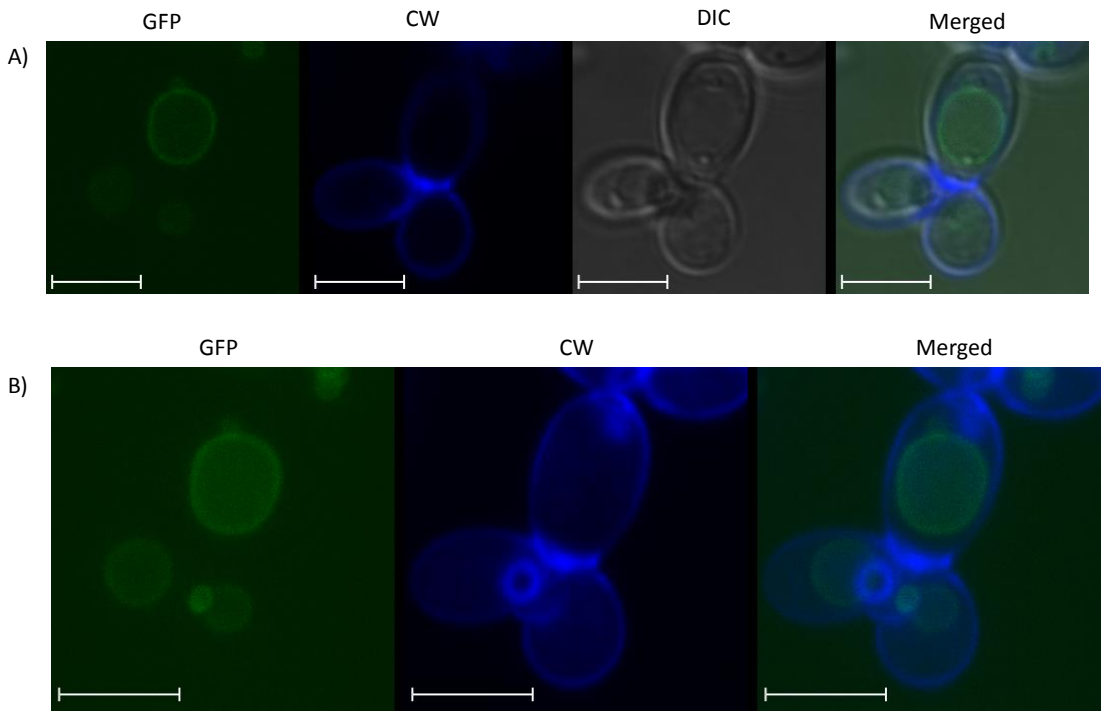


Fig. 7. Single slice and maximum projection of a confocal microscopy Z-stack of yeast cells expressing Mlt1-GFP.

A single Z-stack section (A) and a maximum Z-stack projection (B) is shown from budding yeast cells of C4GFP. Ring-like green fluorescence in these images indicates the vacuolar membrane localization of the Mlt1-GFP fusion protein. The DIC image in (A) also reveals the large vacuole in the mother cell. The Calcofluor White (CW) blue fluorescence in (B) depicts a bud scar (small ring-like structure). Scale bars: 5 μm.

Spot Assay of *Candida albicans* ABC Transporter Mutants

Plate spot assays are a qualitative method to detect increased susceptibilities of gene disruption mutants towards toxic chemicals included in the agar medium. The following shows the results of a spot assay with the *C. albicans* ABC transporter mutants and controls on agar plates containing $CdCl_2$.

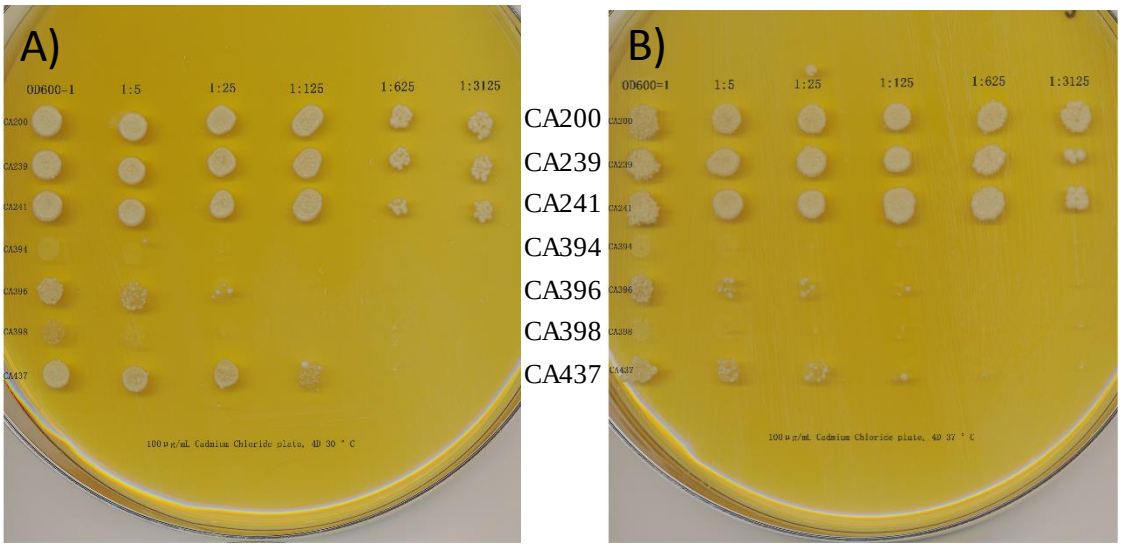


Fig 8. Spot assay with ABC transporter mutants.

Serial dilutions of strains of CA200, CA239, CA241, CA394, CA396, CA398 and CA437 were spotted on agar plates with 100 μg/ml cadmium chloride and incubated at 30℃ (A) and 37℃ (B) for 4 days. The *ycf1/ycf1* mutant CA394 and the *ycf1/ycf1 mlt1/mlt1* double gene disruption mutant CA398 are hypersensitive to $CdCl_2$, while the *mlt1/mlt1* single knock-out strain CA239 does not show this phenotype. Strains CA396 and CA437 harboring a single intact copy of *YCF1* show an intermediate phenotype.

At 30 ℃ and 37 ℃ the *ycf1/ycf1* mutants were highly susceptible to $CdCl_2$. The wild type and *mlt1/mlt1* mutants showed similar resistance to the compound and grew even at the highest dilution of the inoculum. The heterozygous *YCF1* complemented strains showed intermediate susceptibility. Thus, Ycf1p appears to be involved in cadmium resistance.

Agar Disk Diffusion Assays with Cadmium, Mercury, and Lead Compounds

Disk diffusion assay were carried out with $CdCl_2$, $HgCl_2$, and $Pb(CH_3OO)_2$ in order to semi-quantitatively determine the susceptibility of wild-type and ABC transporter mutants to these compounds.

Representative results of cadmium chloride disk diffusion assays are shown in Figure 9 and Table 3.

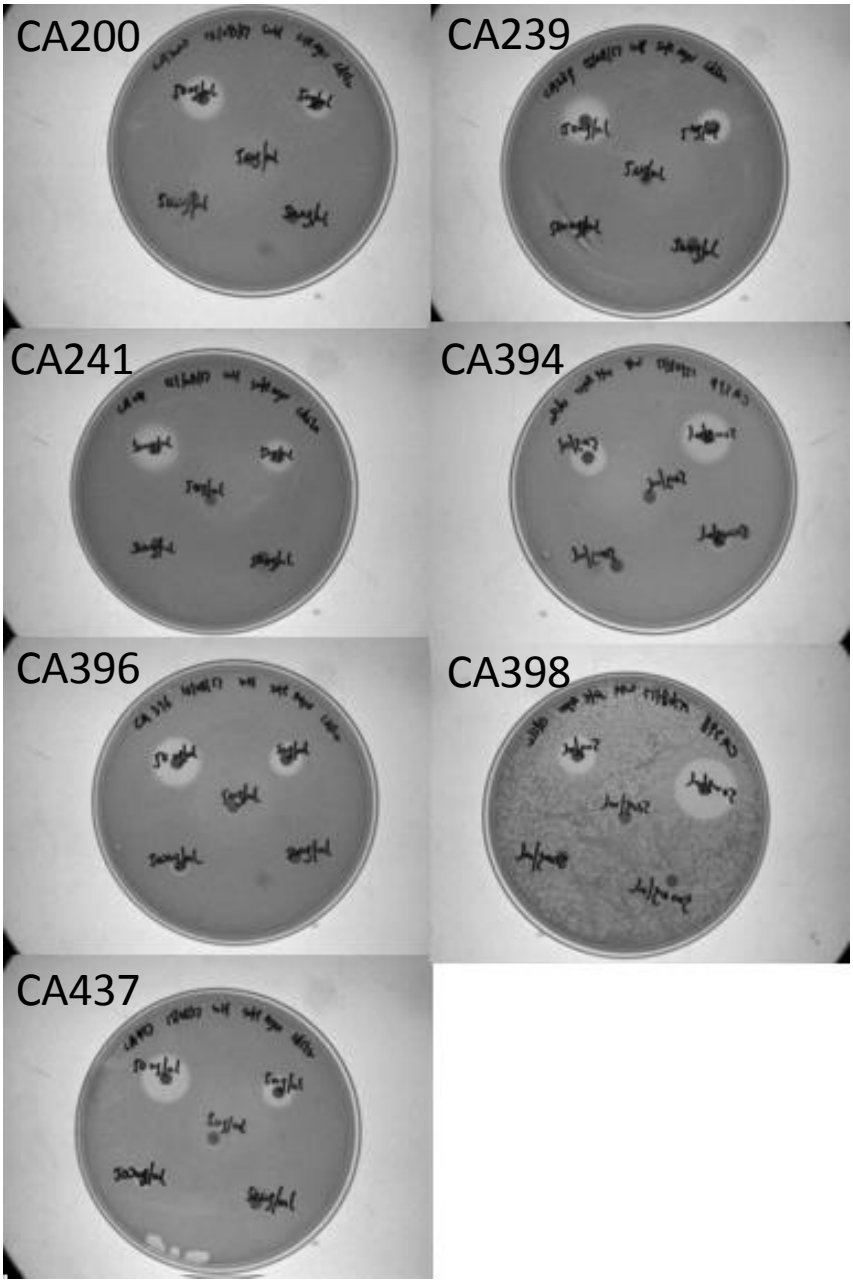


Fig. 9. $CdCl_2$ disk diffusion assay. Strains CA200, CA239, CA241, CA394, CA396, CA398, CA437 were inoculated at OD_{600}= 0.01 in a 0.7% soft agar overlay. Disk loaded with 10 µl of 50mg/ml, 5mg/ml, 500 µg/ml, 50 µg/ml, 5 µg/ml of cadmium chloride solution were deposited on the soft agar. Plates were incubated at 30 ℃ for 24 hours.

Table 3. Diameters of inhibition zones in $CdCl_2$ disk diffusion assays after 24 hours.

$CdCl_2$ concentration used					
Strain	**50 mg/ml**	**5 mg/ml**	**500 µg/ml**	**50 µg/ml**	**5 µg/ml**
CA200	1.9 cm	1.3 cm	-	-	-
CA239	2.3 cm	1.6 cm	-	-	-
CA241	2.2 cm	1.4 cm	0.9 cm	-	-
CA394	2.4 cm	1.8 cm	1.0 cm	0.7 cm	-
CA396	2.3 cm	1.4 cm	0.9 cm	0.7 cm	-
CA398	2.8 cm	1.9 cm	-	-	-
CA437	2.4 cm	1.7 cm	0.8 cm	-	-

The results of the disk diffusion assay suggest that CaYcf1 and to lesser extent Mlt1 are involved in cadmium chloride resistance.

Representative results of mercuric chloride disk diffusion assays are shown in Figure 10 and Table 4.

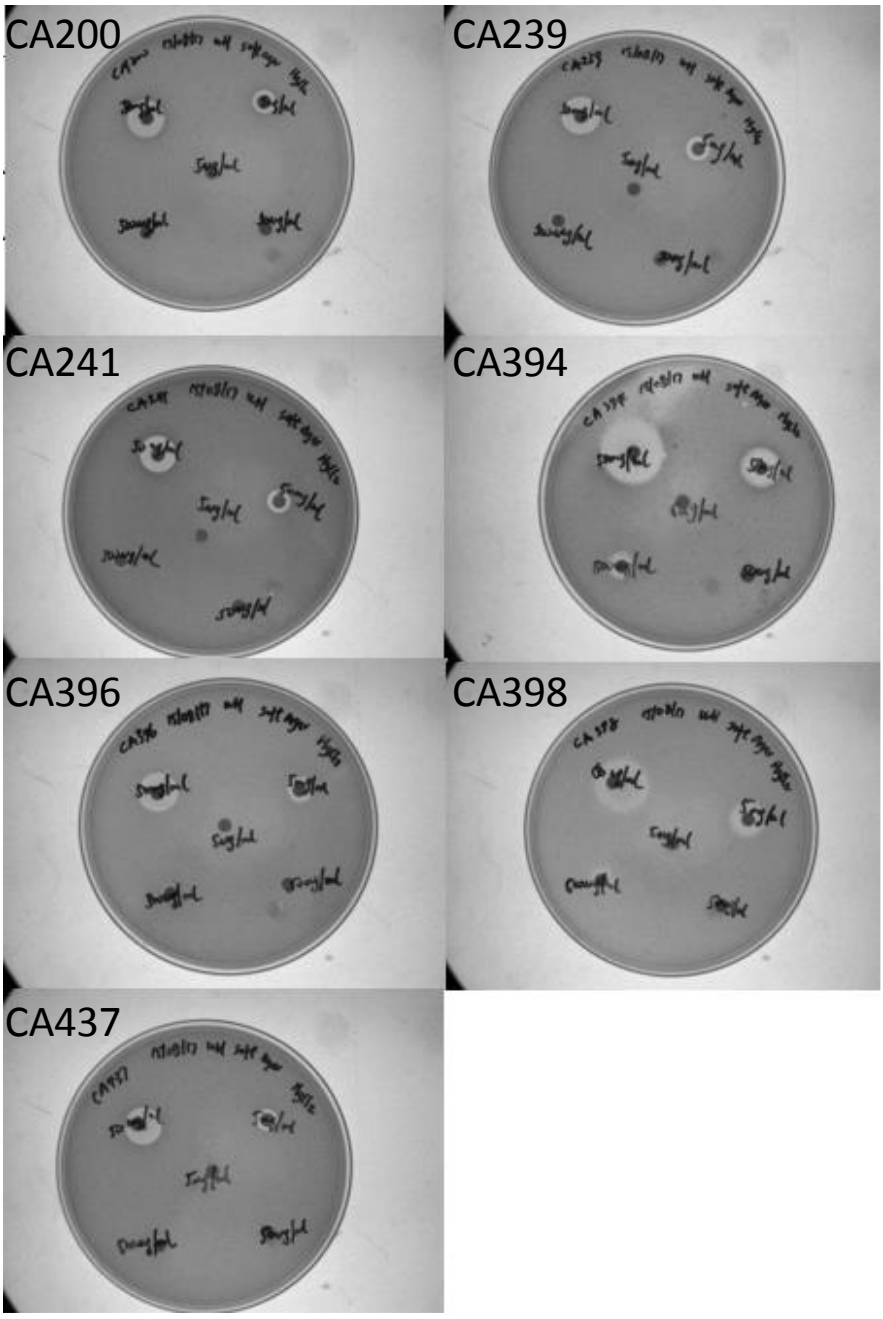


Fig. 10. $HgCl_2$ disk diffusion assay. Strains CA200, CA239, CA241, CA394, CA396, CA398, CA437 were inoculated at OD_{600}= 0.01 in a 0.7% soft agar overlay. Disk loaded with 10 µl of 50mg/ml, 5mg/ml, 500 µg/ml, 50 µg/ml, 5 µg/ml of mercuric chloride solution were deposited on the soft agar. Plates were incubated at 30 ℃ for 24 hours.

Table 4. Diameters of inhibition zones in $HgCl_2$ disk diffusion assays after 24 hours

$HgCl_2$ concentration used					
Strain	**50 mg/ml**	**5 mg/ml**	**500 µg/ml**	**50 µg/ml**	**5 µg/ml**
CA200	1.8 cm	1.1 cm	0.9 cm	-	-
CA239	1.9 cm	1.1 cm	0.9 cm	-	-
CA241	1.7 cm	1.2 cm	0.9 cm	0.8 cm	0.7 cm
CA394	3.0 cm	1.9 cm	1.3 cm	-	-
CA396	1.9 cm	1.2 cm	0.8 cm	0.7 cm	-
CA398	2.1 cm	1.2 cm	0.9 cm	0.7 cm	-
CA437	1.8 cm	1.1 cm	0.8 cm	-	-

The results of the $HgCl_2$ disk diffusion assay suggest that Ycf1 is involved in cadmium resistance. Interestingly, the double mutant *ycf1/ycf1 mlt1/mlt1* CA398 appeared to be less susceptible to mercuric chloride than the single *ycf1/ycf1* mutant CA394.

$Pb(CH_3OO)_2$ disk diffusion assay with strains CA200, CA239, CA241, CA394, CA396, CA398, CA437 were conducted in similar manner as the aforementioned assays. However, disks loaded with 10 µl of 50 mg/ml, 5 mg/ml, 500 µg/ml, 50 µg/ml, 5 µg/ml of lead (II) acetate solution did not yield inhibition zones with the wild-type or mutant strains. Thus this compound appeared to have no effect on *Candida* growth or it did not diffuse into the medium.

Broth Microdilution Assays with $CdCl_2$, $HgCl_2$

Broth microdilution assays were used to assess the susceptibility of the ABC transporter mutants to $CdCl_2$ and $HgCl_2$ in SD medium.

Figure 11 shows the results of the microdilution assay using $CdCl_2$ as growth inhibitor and Figure 12 shows the broth microdilution results with $HgCl_2$.

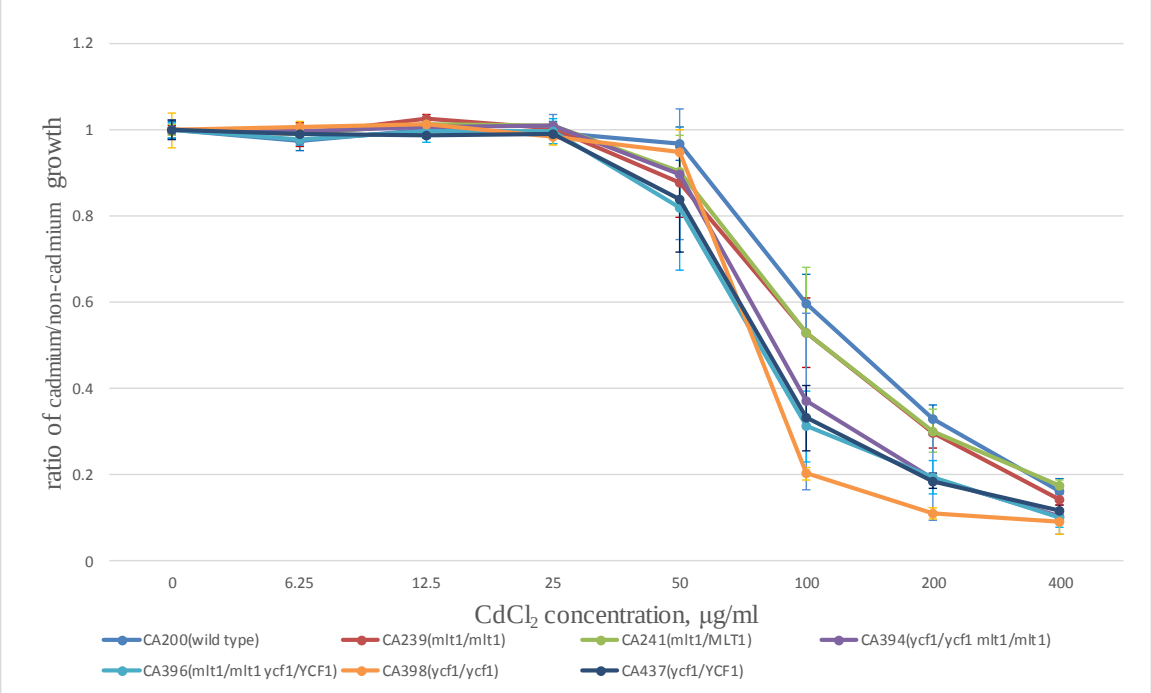


Fig. 11. Growth inhibition of the *C. albicans* strains in $CdCl_2$ microdilution assays. Growth of the homozygous and heterozygous *ycf1* mutants CA394, CA396, CA398 and CA437 mutants is decreased at 100 µg/ml of $CdCl_2$, (but not significant by ANOVA). The results after 24 h of incubation are shown.

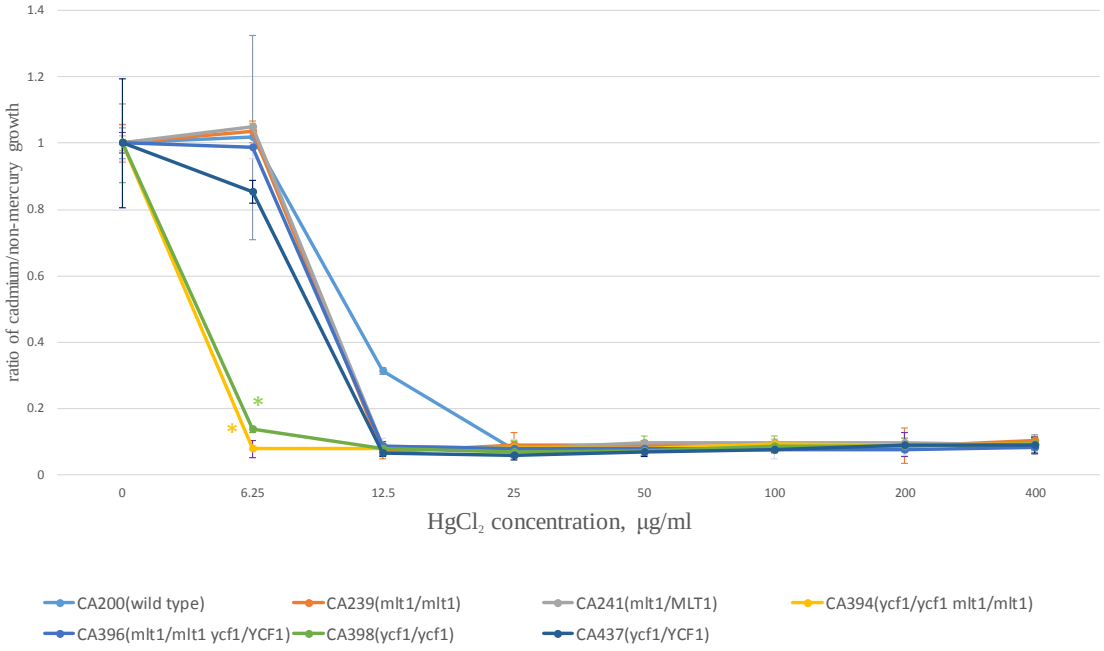


Fig. 12. Growth inhibition of the *C. albicans* strains in $HgCl_2$ microdilution assays. Growth of homozygous *ycf1/ycf1* mutants CA394 and CA398 mutants is substantially decreased already at 6.25 µg/ml of $HgCl_2$ (ANOVA, $p<0.05$). Heterozygous *ycf1/YCF1* mutants and *MLT1* mutants reveal growth reduction at 12.5 µg/ml of $HgCl_2$, which may indicate that losing one allele of *YCF1* or *MLT1* could increase susceptibility to $HgCl_2$. The results after 24 h of incubation are shown.

Broth Microdilution Assay with Fluconazole

In order to assess whether *C. albicans ycf1* and/or *mlt1* mutations increase susceptibility to the antifungal drug fluconazole, broth microdilution assays were conducted similar to the assays described above. Figure 13 shows the results of the microdilution assays with fluconazole.

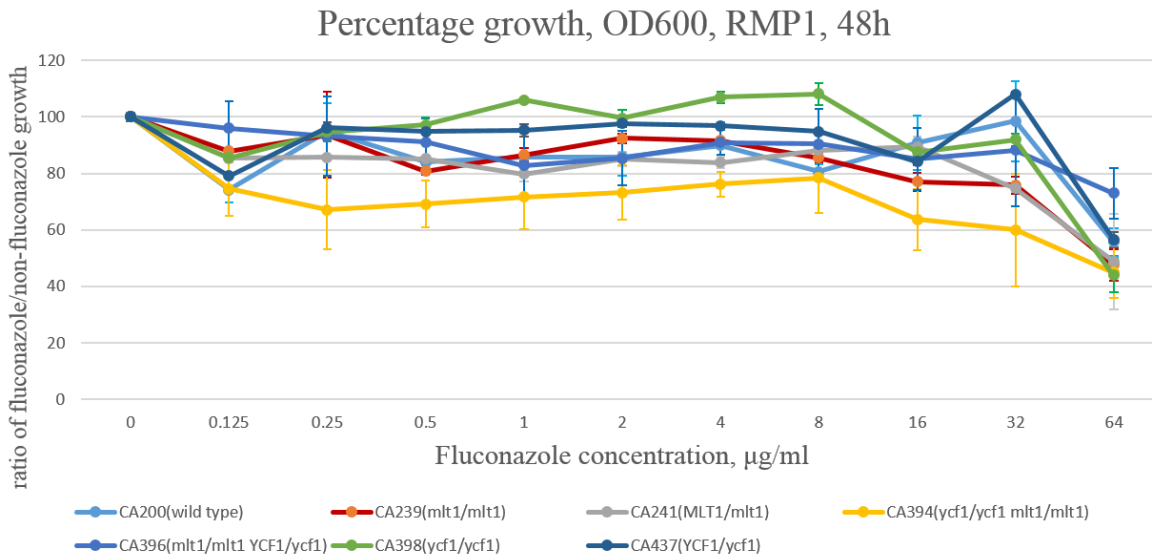
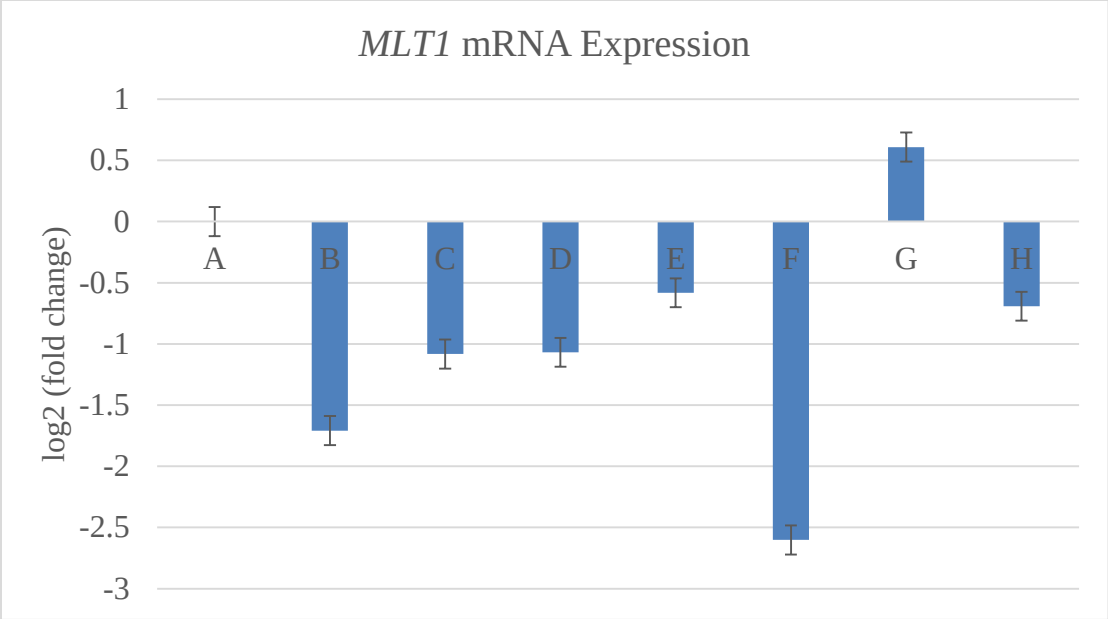


Fig. 13. Growth inhibition of the *C. albicans* strains in fluconazole microdilution assays. Results indicate no significant differences in fluconazole resistance (up to 64 µg/ml) between wild-type and the vacuolar ABC transporter mutants. Results after 48h incubation are shown.

RT-qPCR of *CaYCF1* and *MLT1* mRNA Expression in Presence of $HgCl_2$

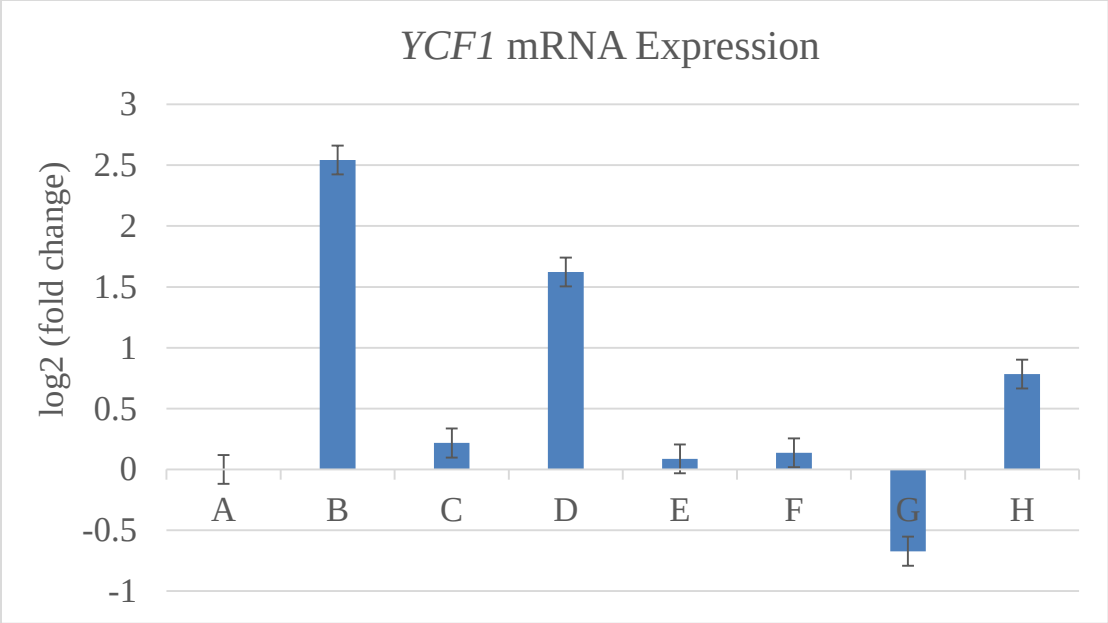
A reverse transcriptase qPCR analysis was conducted to investigate how *MLT1* and *YCF1* mRNA expression in wild-type and mutant *C. albicans* strains is affected by the presence of mercuric chloride. Figures 14 and 15 show representative results of these experiments.

MLT1 expression is generally reduced during presence of the salt, especially in cells lacking *YCF1*. On the other hand, *YCF1* appears to be induced by $HgCl_2$.



	A	B	C	D	E	F	G	H
Strain name	CA200	CA200	CA241	CA241	CA398	CA398	CA437	CA437
Geno type	Wild type	Wild type	*mlt1/MLT1*	*mlt1/cMLT1*	*ycf1/ycf1*	*ycf1/ycf1*	*ycf1/YCF1*	*ycf1/YCF1*
Mercury	-	+	-	+	-	+	-	+

Fig. 14. *MLT1* mRNA expression in *C. albicans* strains during $HgCl_2$ exposure. Results of the RT-qPCR with the wild-type strain CA200, the *mlt1/MLT1* mutant CA241, the homozygous *ycf1* mutant CA398, and the *YCF1*-complemented strain with and without exposure to $HgCL_2$ are shown. CA200 *MLT1* expression without $HgCL_2$ exposure was used as the calibrator. The expression of *MLT1* appears to be reduced by the presence of $HgCL_2$, especially in the wild-type and the complete *ycf1* knock-out. CA241 shows reduced expression because of the defective *mlt1* allele.



	A	B	C	D	E	F	G	H
Strain name	CA200	CA200	CA239	CA239	CA241	CA241	CA437	CA437
Geno type	Wild type	Wild type	*mlt1/mlt1*	*mlt1/mlt1*	*mlt1/MLT1*	*mlt1/MLT1*	*ycf1/YCF1*	*ycf1/YCF1*
Mercury	-	+	-	+	-	+	-	+

Fig. 15. *YCF1* mRNA expression in *C. albicans* strains during $HgCl_2$ exposure. Results of the RT-qPCR with CA200, the *mlt1/mlt1* mutant CA239, the homozygous *mlt1* mutant CA239, the *MLT1* (CA241) and the *YCF1* complemented strain CA437 with and without exposure to $HgCl_2$ are shown, CA200 *YCF1* expression without $HgCL_2$ exposure was used as the calibrator. Exposure to the salt increases expression of *YCF1*, with the exception of CA241. CA437 shows reduced *YCF1* expression due the absence of an

YCF1 allele. Nevertheless, expression of the remaining intact allele appears to be increased during mercury exposure.

In summary, the results of this study showed that the ABC transporter encoded by the *C. albicans* gene *CaYCF1* does confer resistance to cadmium and mercury ions. Consequently, *CaYCF1* also is induced by presence of mercuric chloride. The related ABC transporter Mlt1p does not show major involvement in cadmium and mercury resistance, nor does it appear to be directly induced by mercuric chloride. Furthermore, evidence was provided that, like Mlt1p, the *CaYCF1*-encoded ABC transporter localizes to vacuolar membranes in *C. albicans* yeast, pseudohyphal, and hyphal forms.

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CHAPTER V

CONCLUSIONS

There are at least 18 ABC transporters encoded in the *Candida albicans* genome, but only a few of them have been well characterized [53]. Our study was focused on two *C. albicans* ABC transporters, CaYcf1p and Mlt1p. The CaYcf1p orthologue ScYcf1p was first discovered in *Saccharomyces cerevisiae* and has been studied in detail in this yeast [57]. However, *S. cerevisiae* as a non-pathogenic fungus could not instruct us on the potential role of this ABC transporter in virulence. Furthermore, as a vacuolar membrane transporter, most of Ycf1p's function should be related to the role of vacuoles in fungal cell biology. Vacuoles are important organelles that have crucial intracellular functions such as sequestration of toxic chemicals as glutathione-conjugated complexes and involvement in reproduction via budding in yeasts or in filamentous growth which is a characteristic processes in molds and some yeast-like fungi [39, 78]. Thus, this study's goal was to initiate the functional characterization of CaYcf1p in the pathogenic yeast *C. albicans* which is, in contrast to *S. cerevisiae*, capable of growing in various filamentous forms, i.e. pseudohyphae and true hyphae.

The vacuolar ABC transporter Mlt1p on the other hand was characterized first in *C. albicans* by Theiss and coworkers [6] – the existence of a true orthologue of this transporter in *S. cerevisiae* is unclear. *C. albicans MLT1* gene deletion affected lipid

homeostasis, endocytosis, oxidoreductase activities, and hyphal development [7]. The deletion mutants also revealed a virulence defect because they were unable to invade liver tissue during peritoneal infection of mice [6].

One goal of the current study was the determination of the cellular localization of the CaYcf1p transporter. The C-terminal GFP fusion proteins of CaYcf1p expressed by the strain *C albicans* 6748GFP as well as Mlt1p-GFP expressed by C4GFP were detected by light microscopy in vacuolar membranes; thus, confirming that both transporters likely are occupying similar intracellular sites. The vacuolar membrane localization of CaYcf1p also is consistent with the localization of the orthologous protein ScYcf1p in *S. cerevisiae* [56, 57]. The employment of C-terminal fusions instead of N-terminal fusion constructs, which are more likely to be defective in vacuolar targeting, appears to be a successful strategy to elucidate the localization of these ABC transporters in intracellular membranes. Interestingly, Mason and Michaelis have shown that the N-terminal extension domain of the MRP-like ScYcf1p is crucial for targeting to the vacuole [55]. The GFP-fusion protein strategy was successful in yeast, pseudohyphal, and hyphal forms of the fungi (see Figures 1 to 7). The predominance of smaller vacuoles in the Ycf1p-GFP mutant 6478GFP-1 could indicate that this mutant exhibits reduced tendency to form larger vacuoles. This could also be indicative for functional impairment of the GFP-labeled allele in combination with a putative involvement of CaYcf1p in vacuole fusion as it was reported for the *S. cerevisiae* orthologue ScYcf1 [38, 62]. Future studies, will take advantage of the complete *CaYCF1* gene knock-out to investigate further the role of CaYcf1p in vacuole fusions in *C. albicans*.

Another interesting finding are the tracks of Ycf1p-GFP fluorescence between vacuoles (see pseudohypha in Figure 4). These tracks could represent tubular connections between individual vacuoles in growing cells that lead to propagation of vacuoles along the expanding filaments. Again, this could indicate that CaYcf1 is involved in vacuolar fusion and trafficking. Previous studies have demonstrated that vacuoles are required for cell-cycle progressing and daughter cells will receive organelles from their mother cells [79]. During the yeast cell budding process, the mother cell vacuole will start to extend portions into the emerging bud [78]. In the *C. albicans* Ycf1p-GFP fusion strain mother cells of germ tubes (Figure 6) are full of small vacuoles that might be trafficking into the expanding germ tube to form new, larger vacuoles.

In addition to the localization experiments, several approaches to determine the role of CaYcf1p in detoxification of toxic metal ions such as cadmium, mercury and lead ions were used in this study. Gene disruption mutants of *MLT1* and *CaYCF1* and their associated complemented strains with single intact alleles were crucial in the comparison with wild-type susceptibility to these toxic chemicals. A spot assay using wild-type and mutant strains revealed clear hypersensitivity to $CdCl_2$ in *ycf1/ycf1* mutants (see Figure 8; CA394 and CA398) that was partially complemented by introduction of a single intact *YCF1* allele in the complemented strains (CA437 and CA396). In this assay, no differences were detected after incubation at 30 ℃ and 37 ℃. Because of the increased tendency of *C. albicans* for filamentous growth at elevated temperature, future experiments will explore whether there are disparate susceptibilities in the wild-type and mutant strains during growth in pure yeast, pseudohyphal and hyphal forms.

Most studies on the functions of Ycf1p focused on the yeast like *S. cerevisiae* [56, 61, 64] which does not form true hyphae; therefore, studies in pleomorphic and pathogenic fungus *C. albicans* could reveal novel insights into the role of Ycf1p in detoxification during exposure to environmental conditions that favor filamentous growth, including those conditions in the mammalian host.

The results of this study, revealed no substantial association of the Mlt1 ABC transporter with cadmium and mercury detoxification. Further studies employing different media and growth conditions are needed to confirm this notion. CaYcf1p on the other hand appears to be clearly involved in the detoxification of the toxic compounds, confirming CaYcf1 as the true orthologue of ScYcf1p which has been shown to exert these activities in *S. cerevisiae* according to lots of earlier studies.[56-58, 60]. The disk diffusion assays and broth microdilution assays performed in our study further confirm the role of CaYcf1p in resistance to toxic cadmium and mercury compounds (see Figures 9-12; Tables 3 and 4). The failure of lead (II) acetate to inhibit growth of *C. albicans* in the disk diffusion assay requires further investigation, possibly using different approaches or lead compounds. Potential reasons for this failure could be impaired agar diffusion or restricted access of lead (II) acetate to the intracellular environment [80]. The notoriety of lead as environmental toxin certainly warrants further study [81]. Lead is likely to be harmful to *C. albicans* because of its ability to bind sulfate groups and to interfere with the structural organization of zinc fingers in enzymes [82].

One of the most important functions of ABC transporters in the plasma membrane is drug efflux, on cause for antifungal drug resistance [71]. Multidrug resistance-associated protein 1 (Mrp1) in humans also belongs to ABCC subfamily. A previous study showed

that Mrp1 can complement an *ycf1* defect in yeast and restore transport of glutathione conjugate complexes [61]. For this reason, we tried to test whether Ycf1p and Mlt1p are involved in resistance to fluconazole. However, we did not find convincing evidence that these ABC transporters play a role in fluconazole resistance (Figure 13) . Previous research showed that Mlt1p is involved antifungal drug resistance [7]. The wild-type strain used in this study appeared to exhibit high levels of fluconazole resistance; thus, any potential effect of the vacuolar transporters could be masked by the strong activities of plasma membrane efflux pumps [83, 84].

The RT-qPCR expression analyses in this study revealed that *CaYCF1* expression levels increased when mercuric chloride was present, further confirming Ycf1p's role in mercury detoxification (see Figures 14 and 15). Decrease of *MLT1* expression during exposure to the salt could be caused by a general downregulation of gene expression due to the toxi effects of the compound – this was especially pronounced in *ycf1* mutants that apparently lack a major mercury detoxification mechanism. How potential vacuolar fusion or trafficking defects in these mutants might affect detoxification remains to be elucidated. Our single and double ABC transporter disruption mutants could be valuable tools to explore details of the detoxification and gene regulation processes.

In conclusion, the initial functional characterization of the *C. albicans* vacuolar membrane ABC transporter CaYcf1p in this study has confirmed that this protein is indeed the homologue of ScYcf1p in *S. cerevisiae*. However, the fact that this ABC transporter is situated in a pathogenic fungus with multiple morphologies, has opened new lines of investigation towards its role in filamentous growth, vacuole inheritance, and virulence of *C. albicans.*

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